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Establishment and characterization of DB-1: a leptin receptor-deficient murine macrophage cell line

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Abstract

Metabolic and immune mediators activate many of the same signal transduction pathways. Therefore, molecules that regulate metabolism often affect immune responses. Leptin is an adipokine that exemplifies this interplay. Leptin is the body's major nutritional status sensor, but it also plays a key role in immune system regulation. To provide an in vitro tool to investigate the link between leptin and innate immunity, we immortalized and characterized a leptin receptor-deficient macrophage cell line, DB-1. The cell line was created using bone marrow cells from leptin receptor-deficient mice. Bone marrow cells were differentiated into macrophages by culturing them with recombinant mouse macrophage colony stimulating factor, and passaged when confluent for 6 months. The cells spontaneously immortalized at approximately passage 20. Cells were cloned twice by limiting dilution cloning prior to characterization. The macrophage cell line is diploid and grows at a linear rate for 4–5 days before reaching the growth plateau. The cells are MAC-2 and F4/80 positive and have phagocytic activity similar to primary macrophages from wild-type and leptin receptor-deficient mice. DB-1 cells were responsive to stimulation with interferon-γ as measured by increase in *Nos2* transcript levels. In addition, DB-1 macrophages are not responsive to the chemotactic signaling of adipocyte conditioned media nor leptin when compared to primary WT macrophages. We believe that DB-1 cells provide a dependable tool to study the role of leptin or the leptin receptor in obesity-associated inflammation and immune system

dysregulation.

Keywords: Macrophages, Leptin receptor, Immortal cell line

Introduction

Leptin, the product of the *ob* gene, is a 16 kDa peptide hormone predominantly secreted by white adipocytes (Ahima and Flier 2000; Zhang et al. 1994). Leptin is structurally similar to type I cytokines and referred to as an adipokine or adipocytokine (Otero et al. 2005). Leptin signals through its receptor (LepR) encoded by the *Lepr* gene (Chen et al. 1996). The LepR exists in six alternatively spliced isoforms (Lee et al. 1996). The long isoform of LepR is a member of the interleukin-6 receptor family of class 1 cytokine receptors, and is responsible for most of leptin's physiological activities (Baumann et al. 1996; Tartaglia et al. 1995).

Leptin is involved in a plethora of physiological actions, and plays a major role in the regulation of neuroendocrine function and energy homeostasis (Chan et al. 2003). Leptin stimulates the production of anorectic neuropeptides and suppresses the action of orexigenic peptides in the arcuate nucleus of the hypothalamus (Cheung et al. 1997; Schwartz et al. 1997; Thornton et al. 1997), where LepR is highly expressed (Elmquist et al. 1998). In addition, leptin exerts other physiological effects that impact reproduction, hematopoiesis, angiogenesis and immunity (Wauters et al. 2000). Leptin's effects are exerted through the expression of LepR on different types of tissues and cells throughout the body; these are often referred to as leptin's peripheral effects. Furthermore, leptin plays an important role in the regulation of both innate and adaptive immunity (La Cava and Matarese 2004; Matarese et al. 2005), and different immune cells express LepR, which supports the notion that leptin plays a direct role in immune function (Matarese et al. 2005). T-cells (Batra et al. 2009), B-cells (Papathanassoglou et al. 2006), neutrophils (Caldefie-Chezet et al. 2003), monocytes (Raso et al. 2002), dendritic cells (Mattioli et al. 2005) and NK cells (Tian et al. 2002) all express LepR, and leptin has been shown to modulate their functions in vivo and in vitro (Papathanassoglou et al. 2006).

Leptin induces diapedesis of monocytes and macrophages through an epithelial layer in vitro and the production of pro-inflammatory cytokines from both cell types (Curat et al. 2004; Gruen et al. 2007). In macrophages, leptin augments LPS-induced cytokine secretion (Gainsford et al. 1996; Loffreda et al. 1998) via IRAK-1 up-regulation, (Vaughan and Li 2010) and potentiates IFN-γ-induced expression of nitric oxide synthase (Raso et al. 2002).

Non-genetic obesity is characterized by a hyperleptinemic state (Considine et al. 1996) and a compromised immune system (Falagas and Kompoti 2006). In addition, there is increased pro-inflammatory macrophage recruitment in the adipose tissue of obese individuals (Weisberg et al. 2003; Xu et al. 2003), and "obese" macrophages have impaired phagocytic activity (Krishnan et al. 1982; Mancuso et al. 2002).

It remains unclear what promotes a proinflammatory state in obese animals and people and their higher susceptibility to infections. Our central hypothesis is that leptin contributes directly to this problem by regulating macrophage function. Our objective here was to create a continuous macrophage cell line to allow for investigation of LepR's impact on macrophage function. We detail the properties of this new leptin receptor-deficient macrophage cell line, DB-1 in this paper.

Materials and methods

Animals

Male leptin receptor-deficient (*db/db*) mice as well as gender- and aged-matched wild-type (WT) C57BL/6 mice controls were purchased from Jackson Laboratories (Bar Harbor, ME). All animal procedures were performed with prior approval and regular monitoring of the Institutional Animal Care and Use Committee (IACUC) at Kansas State University.

Reagents

Biomedium was prepared using Dulbecco's Modified Eagle's Medium (DMEM, Atlanta Biologicals, Lawerenceville, GA, USA) supplemented with 5 % Nu Serum (Collaborative Biomedical Products, Bedford, MA, USA), 5 % fetal bovine serum (FBS, Atlanta Biologicals), 10 % Opti-MEM reduced Serum Medium (Invitrogen, Carlsbad, CA, USA), 50 μg per ml gentamicin (Atlanta Biologicals; DMEM10) and 1.5 ng per ml recombinant mouse M-CSF (rmM-CSF) (R&D Systems, Minneapolis, MN, USA). Cell culture medium was prepared using DMEM supplemented with 10 % Opti-MEM, 15 mmol/L HEPES and 100 μg per ml gentamicin (Sigma-Aldrich, St Louis, MO, USA). Recombinant mouse leptin (rm-leptin) and IFN-γ were purchased from R&D Systems Inc. Fluorescent spheres were purchased from Invitrogen. Phorbol myristate acetate (PMA; Sigma-Aldrich) was dissolved in DMSO at a concentration of 1 mg/ml and aliquoted at 10 μg/ml concentrations and stored at −80 °C. Cells were stimulated with 10 μl of freshly thawed aliquots.

Collection of peritoneal macrophages

Peritoneal exudate macrophages from WT and *db/db* mice were induced by *i.p.* injection of 1.5 ml of sterile, 2.9 % thioglycollate (DIFCO, Detroit, MI, USA). Four days after injection, mice were anesthetized via isoflurane (IsoFlo, Abbott, Abbott Park, IL, USA) inhalation and euthanized via cervical dislocation. Peritoneal exudate macrophages were collected by washing the peritoneal cavity twice with 12 ml of ice-cold PBS.

Differentiation of Bone Marrow Derived Macrophages

Bone marrow cells from leptin receptor-deficient mice (*db/db*) were collected by flushing the humeri, tibiae and femora as previously described (Armstrong et al. 1993). The undifferentiated cells were resuspended in biomedium containing rmM-CSF (Metcalf et al. 1982), needed for macrophage differentiation, counted and plated at a density of 3–5 × 10^6 cells per 100-mm dish. Cells were incubated at 37 °C in 8 % CO_2 and non-adherent cells were removed. Cells were adherent and were dispersed with trypsin/EDTA (10 % trypsin, 0.1 mM Na_2EDTA; Atlanta Biologicals) to passage the cells. Cells were passed twenty times until spontaneous immortalization occurred as was described previously (Beharka et al. 1998). For activation and phagocytosis assays, bone marrow cells from *db/db* and WT mice, and for cytokine assays bone marrow WT mouse cells were allowed to mature for 7–10 days in biomedium before primary macrophages were used.

Limiting dilution cloning of cell lines

Cells were dispersed with trypsin/EDTA as described above and resuspended in DMEM10. Cells were counted and each dilution was plated in 48 wells of a Costar 96-well, flat-bottom tissue culture plate. A 1:10 serial dilution scheme was used starting at 100 cells/well in 100 µl DMEM10 and ending at 0.01 cell/well. Cells were then incubated at 37 °C in 8 % CO_2. Only clones that grew from dilutions having less than a 30 % positive growth were selected and expanded. The limiting dilution was repeated twice to ensure pure clones. Two final clones were selected and characterization experiments were performed using these clones. The cells were confirmed to be of mouse origin by positive PCR of mouse *Actb* gene and the positive staining of the cells for mouse-specific MAC2 antigens between July and August 2009.

Genotyping of cell line

Transcript expression of the long isoform (LepR) of the *Lepr* gene (NCBI 16847) was assessed using qRT-PCR and the following primers: *Lepr* forward 5′-GCA ACC CAC CAT GAT TTC ACC ACA-3′; reverse 3′-AGG ATT CCT GCC TCA CCA GTC AAA-5′. The internal control gene used was β-actin (NCBI number 11461) with the following primers: *Actb* forward, 5′-TGT GAT GGT GGG AAT GGG TCA GAA-3′; *Actb* reverse, 3′-TGT GGT GCC AGA TCT TCT CCA TGT-5′. Fold change in transcript level of each cell type was quantified using the method described by Pfaffl (2001), and the formula using WT macrophages as the reference group:

$$\text{Fold change} = \mathrm{E}^{\Delta ct \text{ target gene (reference - exp)}} / \mathrm{E}^{\Delta \text{ct housekeeping gene (reference - exp)}};$$

where E is the efficiency of the primers, and the housekeeping gene is *ActB* (encodes for β-actin).

Cell cycle determination

Cell cycle determination and ploidy of the cell line was determined using flow cytometry. Briefly, cells in culture were dispersed, collected and counted. A total of 2×10^6 cells were transferred to 12 × 75-mm polystyrene tubes (Falcon, Brookings, SD, USA). Vindelov's propidium iodide (PI) solution was added and cells were incubated for 5–10 min at 4 °C (Vindelov et al. 1983). Ploidy analysis was analyzed with singlet discrimination to detect PI-stained mononuclear cells.

Assessment of cell growth

Cell growth of the leptin receptor-deficient macrophage cell line, DB-1, was determined by counting the cells. DB-1 cells were seeded (5×10^5 cells/well) in 6-well plates. At each time point, (1, 2, 3, 4, 5, 6 days) the medium was aspirated from three plates and the cells were detached with trypsin/EDTA. Live cells were assessed by trypan blue exclusion and counted on a hemacytometer.

Antibody phenotyping of macrophage cell lines and flow cytometry

Macrophage cell line (DB-1), peritoneal macrophages and bone marrow-derived macrophages were probed with antibodies specific for the following surface markers: CD11b, F4/80, MAC-2, Ly6G (e-Bioscience, San Diego, CA, USA) and Ly6C (BD Pharmigen, San Diego, CA, USA) after culture for at least 24 h. Appropriate isotype controls were used for each antibody as the negative control for FACS analysis. Briefly, macrophages were recovered and plated at 1×10^5 cells per well of 96-well tissue culture plates. Fc receptors were blocked by incubating the cells with 50 % goat serum (Atlanta Biologicals) in PBS for 30 min at 4 °C. Antibodies and respective isotype controls were added at the appropriate concentrations and incubated for 1 h at 4 °C. Cells were washed twice with Hank's buffered salt solution (HBSS). Cells were transferred to 12 × 75-mm polystyrene tubes (Falcon), washed once with HBSS and resuspended in 200 µl of 1 % formalin/HBSS. The cells were placed on ice and amount of staining of each marker was assessed by flow cytometry using a FACS Calibur analytical flow cytometer (Becton Dickson, San Jose, CA, USA) measuring 5,000 events per sample. Data analysis was performed with WinList software (Verity Software House, Topsham, ME, USA). The amount of staining of each marker was assessed after subtracting the percent positively stained cells in the isotype control groups.

Giemsa staining of metaphase chromosomes

5×10^6 DB-1 cells were allowed to attach for 8 h in a 100-mm tissue culture plate. Cells were washed and incubated with 10 µg/ml of colchicine (Colcemid, GIBCO-Life Technologies, Grand Island, NY, USA) overnight. Cells were dispersed with trypsin/EDTA as described above, added to 5 ml of PBS and were centrifuged at 350×*g* for 5 min. PBS was removed and the pellet was loosened by gently flicking the base of the tube and 2 ml of freshly made 0.l M KCL were added to the loosened pellet in a drop-by-drop fashion. Two additional ml of 0.1 M KCL were added and the cells

were incubated at 37 °C for 25 min. Cells were fixed with methanol and acetic acid (3:1) by adding 10 drops of fixative (room temperature) to the cell suspension and incubating at 23 °C for 10 min. Cells were gently pelleted at 300×*g* for 5 min and 2 ml of fixative were added to the cells. Cells were resuspended in fixative and were immediately dropped onto tilted glass slides 3–6 drops per slide to optimize cell spreads. Slides were air dried and stained with a 1:50 dilution of Giemsa stain (KaryoMax Giemsa Stain, GIBCO-Life Technologies). Slides were washed by sequential dipping in PBS and water. Cells were air dried, mounted with permount (Fisher Scientific, Fairlawn, NJ, USA) and viewed on a compound microscope and imaged. 50 cells in apparent metaphase arrest were scored for chromosome number and one representative cell was analyzed and presented.

Cytokine secretion

Quantitative enzyme-linked immunosorbent assay (ELISA) using DB-1 macrophage supernatants. DB-1 macrophages were seeded in a 24-well plate (1×10^5 cells/well) and cells were stimulated with IFN-γ (10 U/ml) for 4 h and/or LPS (15 μg/ml) overnight. Supernatants were then collected, centrifuged at 350*g* for 5 min to remove cell debris, and IL-6 cytokine concentrations were determined from freshly collected supernatants.

Phagocytosis assays

Bone marrow-derived, peritoneal and DB-1 macrophages that were in culture for at least 24 h. were dispersed with trypsin/EDTA, recovered, and 1×10^6 cells were seeded per well in 24-well tissue culture plates. Cells were incubated at 37 °C in 8 % CO_2 for 2 h. Cytochalasin D from *Zygosporium mansonii* (Sigma-Aldrich) was added to the negative control plates to inhibit phagocytosis. These plates were incubated at 4 °C for 2 h to further inhibit phagocytosis. Experimental plates were kept at 37 °C. Red fluorescent beads (diameter, 0.2 μm; Invitrogen) were added to each well to a final concentration of 0.1 %. Phagocytosis was stopped after 60 min by centrifuging the 24-well plates at 350×*g* for 5 min, removing the supernatant, and washing twice with PBS. The cells were transferred to 12 × 75-mm polystyrene tubes (Falcon), washed twice with PBS and resuspended in 200 μl of 1 % formalin/PBS. The cells were placed on ice and phagocytosis was assessed by flow cytometry using a FACS Calibur analytical flow cytometer (Becton Dickson) and measuring 10,000 events per sample. The percentage of phagocytosis in the experimental groups was assessed after subtracting the percentage phagocytosis of the negative control treatment group.

Activation of DB-1 with rm-IFNγ

To determine whether DB-1 macrophages respond to IFN-γ in the same fashion as primary macrophages, the DB-1 cells were stimulated with IFN-γ, and expression of *nos2* was assessed using qRT-PCR. Briefly, primary WT, *db/db* and DB-1 macrophages were plated separately in 24-well plates at a density of 1×10^6 cells per well and incubated with or without IFN-γ (100 U/ml, R&D Systems, Minneapolis, MN, USA) for 16 h. RNA was collected using TRI reagent (MRC,

Cincinnati, OH, USA) and further purified with DNAse using the E.Z.N.A Total RNA kit (Omega Bio-Tek, Norcross, GA, USA). Gene expression of *nos2* (NCBI number 18126) was quantified using qRT-PCR with the following primers: *nos2* forward, 5′-CTG CTG GTG GTG ACA AGC ACA TTT-3′; *nos2* reverse, 3′-ATG TCA TGA GCA AAG GCG CAG AAC-5′. *Actb* was quantified using the previously mentioned primers. Fold change in transcript level of each cell type was quantified using the Pfaffl method (2001) as described above. For nitric oxide (nitrite determinations), cells were incubated overnight in 0, 1, 10 and 100 U/ml of IFN-γ. Nitrite concentrations were determine in the supernatant 16 h. later as described previously (Mordica et al. 2009).

Adipocyte collection and conditioned-medium preparation

Adipocytes from epididymal and inguinal fat pads of obese and lean male C57BL/6 mice were collected in cell culture medium, pooled and minced into small pieces. Tissue was digested in Krebs–Ringer Buffer (KRB) containing 1 mg/ml type II collagenase (Sigma-Aldrich) in an orbital shaker for 40 min at 37 °C. The digested tissue was filtered through nylon cell strainers (100 μm; Fisher Scientific, Pittsburg, PA, USA), washed with KRB, and separated by centrifugation for 1 min at 350×*g*. The floating adipocytes were collected and cultured in DMEM at 37 °C, 8 % CO_2 for 24 h. The adipocyte-conditioned medium (ACM) containing all factors released by the adipocytes was collected and used for migration experiments with thioglycollate elicited peritoneal and cell line DB-1 macrophages. Control medium (CM) was obtained using the same protocol but without adding adipocytes. Media were stored at −80 °C until use.

Migration assay

Wild-type peritoneal macrophages were collected as described above and suspended in serum-free culture medium. Cells were placed in the upper chamber of a 5 μm polystyrene filter (6-transwell format; Corning, Lowell, MA, USA). Cells were left to adhere for 3 h; at which point medium in the lower chamber was replaced with either medium (CM, negative control), CM supplemented with 10 ng/ml rm-leptin (ACM), or CM supplemented with 2 μM phorbol 12-myristate 13-acetate (PMA, positive control). After 3 h of incubation, cells that had not migrated and remained in the upper chamber were removed by gently swiping the filters with cotton swabs. Filters were fixed in 2 % ethanol for 5 min. Cells located on the lower side of the filter were stained with Diff-Quick (Dade Diagnostics, Aguada, PR, USA), and counted as migrated cells. These cells were quantified from 5 to 10 fields/condition and cell type.

Statistical analysis

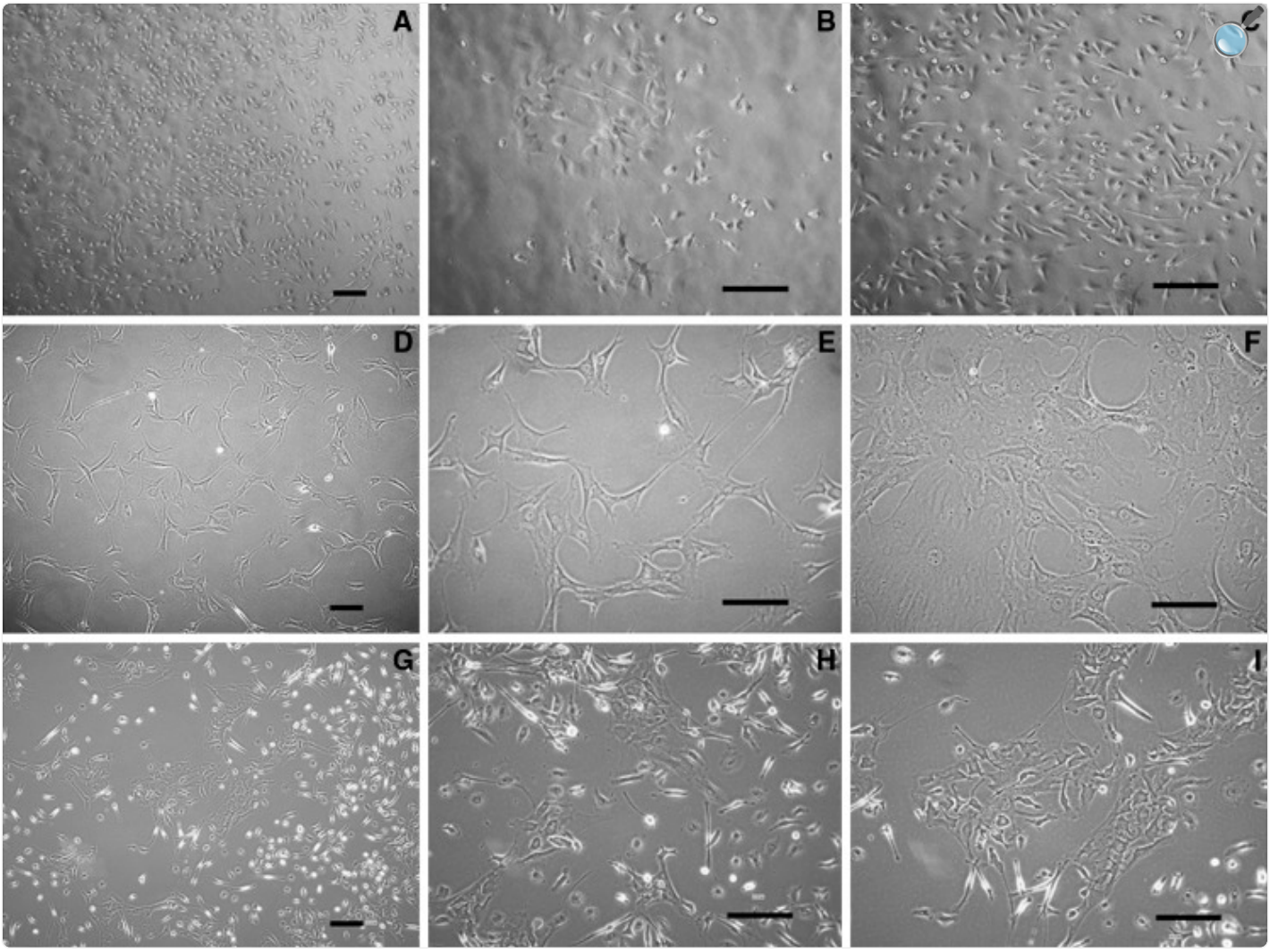
Results are reported as mean ± standard deviation (SD) unless otherwise specified. ANOVA analysis was performed and least significant differences (LSD) were calculated when appropriate. Analysis was done using SPSS software for Windows version 17.0 (SPSS Inc. Chicago, IL, USA). Statistical significance was determined at p value <0.05.

Results

Establishment of DB-1 cell line

We established an immortal leptin receptor-deficient cell line from mouse bone marrow-derived macrophages. Bone marrow cells from leptin receptor deficient mice (*db/db*) were collected and cultured in biomedium as described in the Materials and Methods section. Differentiated macrophages were passaged at confluency. Cells were kept in biomedium for 6 months when the cells appeared to grow at a stable rate after twenty passages. At that time, we did two consecutive limiting dilution cloning procedures. The DB-1 cells were selected from cloned cells from the second cloning. DB-1 cells lost their dependency to M-CSF during the cloning process. They were propagated and shifted to a regular medium, DMEM supplemented with 2 % Nu serum and 2 % FBS. DB-1 cells exhibit morphological properties characteristic of macrophages, and are adherent with multiple processes (Fig. 1). To confirm the genotype of the DB-1 cells as leptin-receptor deficient, we used qRT-PCR to confirm the absence of LepR. Similarly to primary peritoneal *db/db* macrophages, DB-1 had low levels of *Lepr* transcripts, and expression ranged from 0.1 to 12 % of WT macrophages ($p < 0.05$, Table 1).

Fig. 1.



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Morphology of DB-1 cells was determined using light microscopy. Photomicrographs of primary macrophages in culture (**a**–**c**) at low confluency at ×400 (**a**), ×600 magnification (**b**) and at high confluency at ×600 magnification (**c**). DB-1 cells (**d**–**f**) at low confluency at ×400 (**d**), ×600 (**e**) and at high confluency at ×600 magnification (**f**), and 2ASD1.10 macrophage cells (Mordica et al. 2009) in culture (**g**–**i**) at low confluency at ×400 (**g**), ×600 (**h**) and at high confluency at ×600 magnification (**i**). *Scale bar* 12 μm

Table 1.

Relative transcript level of *Lepr* in DB-1 macrophage cells and peritoneal *db/db* macrophages compared to WT peritoneal macrophages

Cell type	Percent expression compared to wild-type macrophages[a]
db/db macrophages	1.4 (0.1–3.5)[b]
DB-1 macrophages	1.1 (0.1–12)[b]

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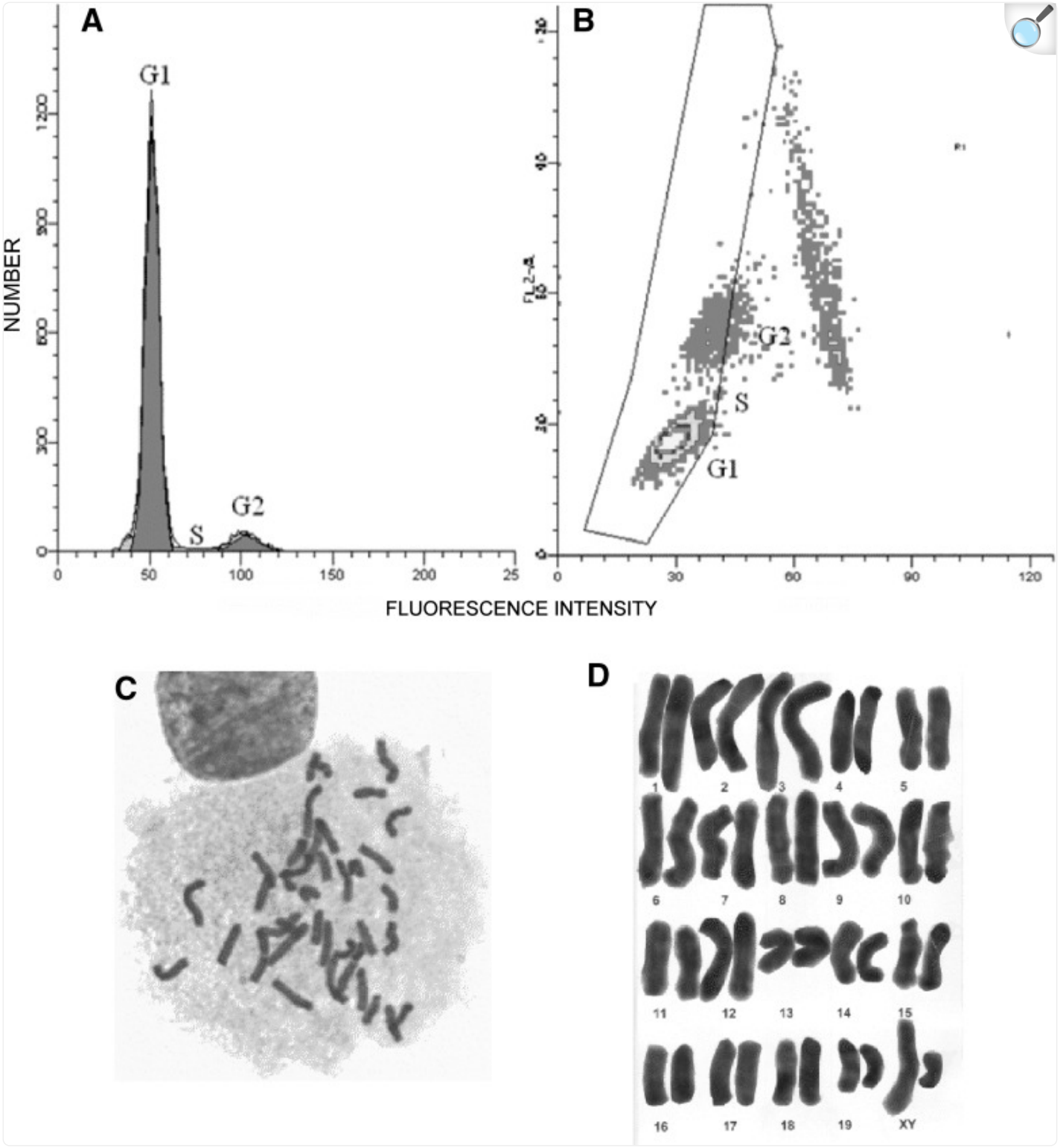
[a]Fold expression was calculated according to Pfaffl (2001) using WT values as control group and *ActB* as the reference gene

[b]Results from 4–6 samples per group, are reported as median (range) expression $p < 0.05$ compared to 100 % expression in WT macrophages

To assess DB-1 ploidy and cell cycle distribution, cell cycle analysis was done using flow cytometry (Vindelov et al. 1983). The DB-1 cell line was homogenous consisting of diploid cells, of which 90–95 % cells were in G1 phase, 3–9 % in G2 phase and 0–2 % in S phase (Fig. 2a, b). 50 metaphase-stage cells (See Fig. 2c for an example) were analyzed for chromosome number and 49 out of 50 cells examined contained the normal complement of 38 chromosomes with one X and one Y chromosome (Fig. 2d); confirming the origin of the cells from a male mouse.

Fig. 2.



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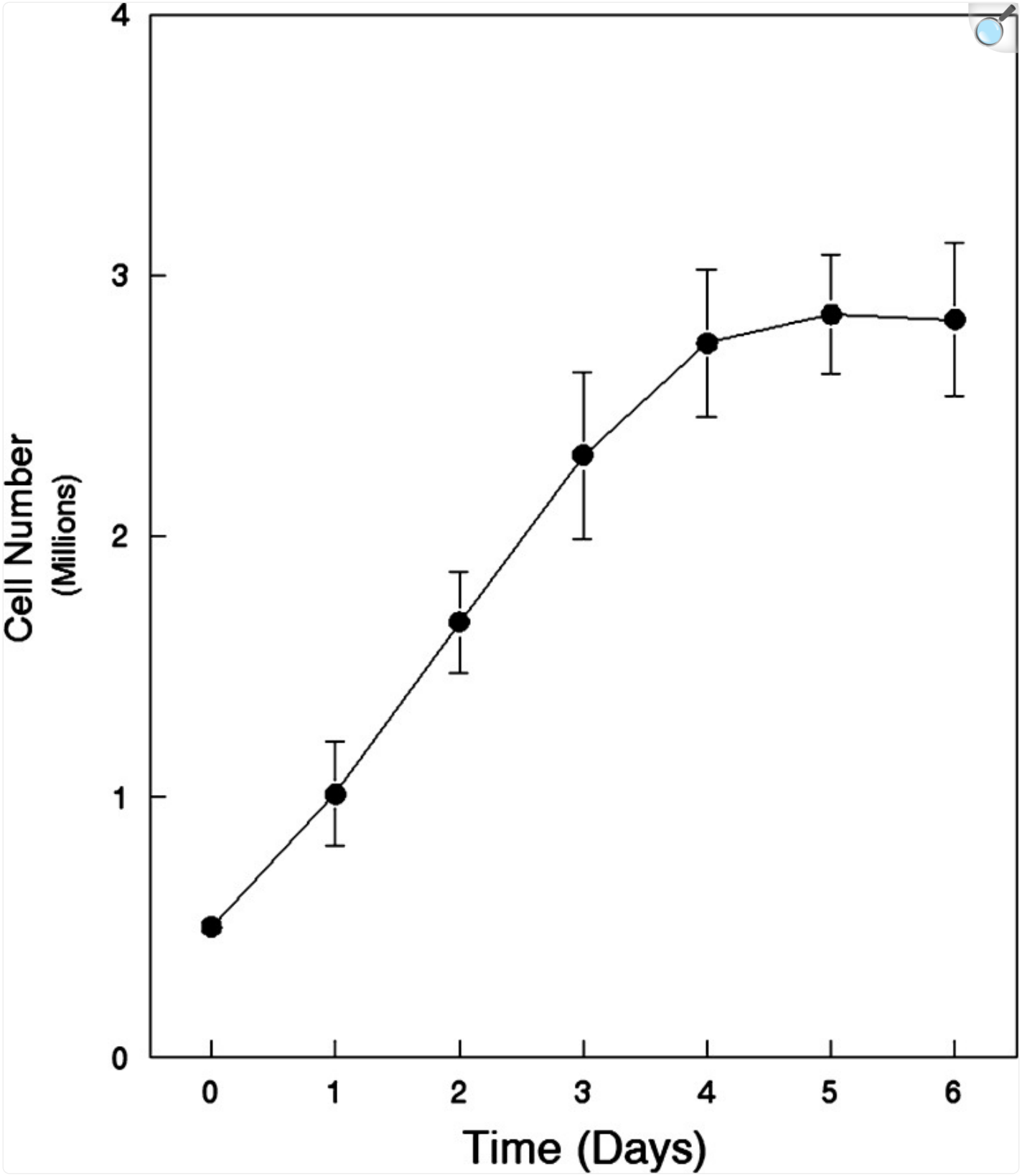
Cell ploidy, DNA cycle and chromosome analysis of the DB-1 cell line. **a** DB-1 DNA histogram. **b** DB-1 *dot*

plot of cell DNA content. **c** metaphase chromosomes of a representative DB-1 cell. **d** Giemsa-stained chromosomes organized by homology, size and shape

DB-1 cell growth was determined and we found that during the linear portion of the growth curve, an average of $5.6 \pm 1.1 \times 10^5$ cells were added to the cell culture each day (Fig. 3). Cell growth plateaued after 4–5 days and the cells became contact inhibited and remained stable for as long as 3 weeks, which was the longest we ran the experiment. The slow growth rate along with the growth arrest at confluency is consistent with a cell line that is non-transformed and contact inhibited (Schuler et al. 1977) but it is unclear how these cells became immortalized. The fact that most cells are in the G1 phase of the cell cycle is consistent with the quiescent nature of the cells. Detailed sequencing of the DB-1 cell genome will be necessary to determine possible causes but these analyses are beyond the scope of this study.

Fig. 3.



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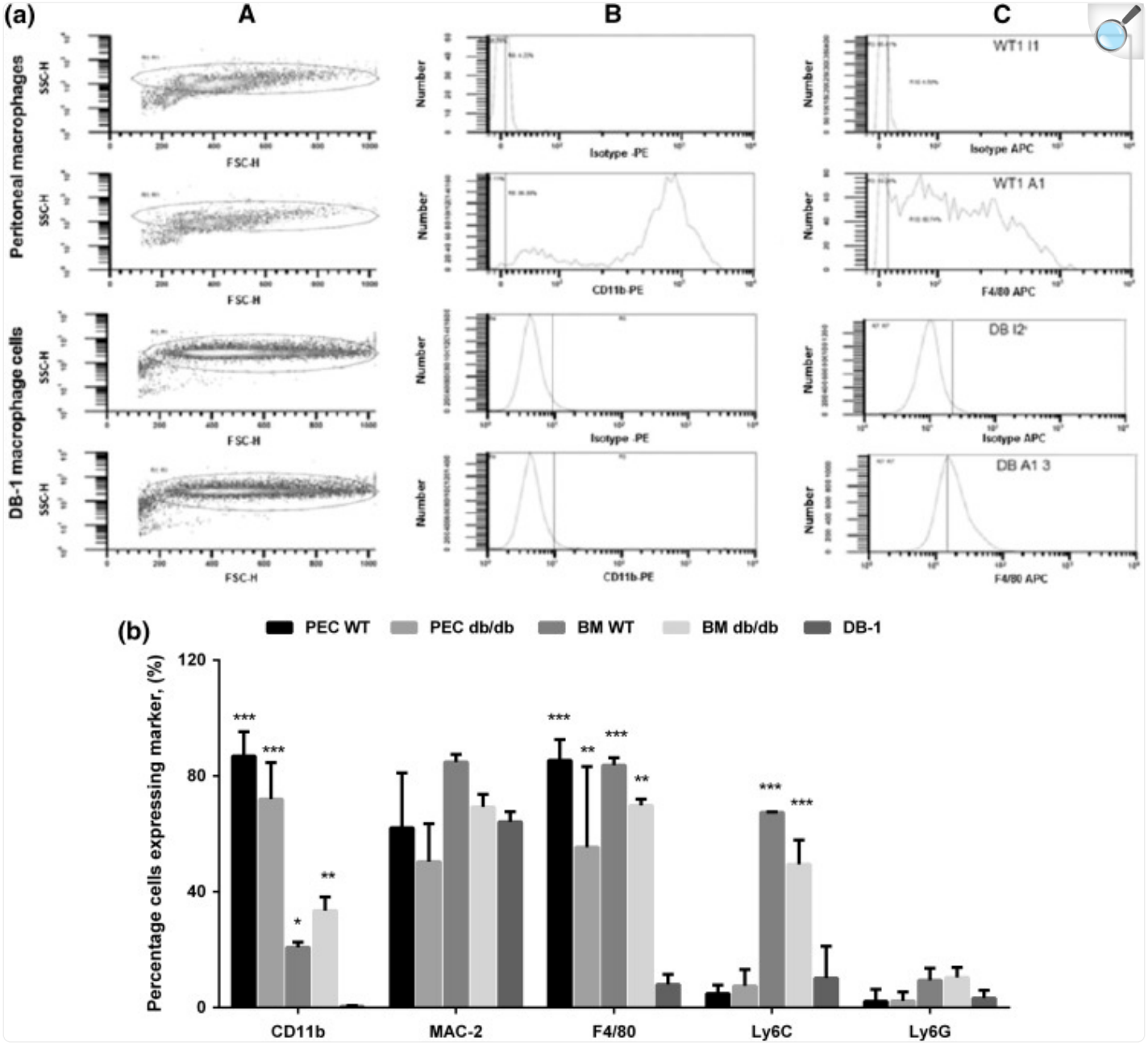
Growth of DB-1 cells. Cell growth was assayed at *0*, *1*, *2*, *4*, *5* and *6*, days after seeding of 5×10^5 DB-1 cells in 6-well plates. Cells were counted as described in the "Materials and methods" section. Each time point value is the mean of two independent experiments ± SD, 3 replicates per experiment

Cell surface phenotype

To verify that DB-1 cells were in the monocyte/macrophage lineage, we compared their cell surface molecule phenotype to primary peritoneal and bone marrow-derived macrophages (Fig. 4a, b). Primary macrophages had similar levels of F4/80 and MAC-2, regardless of their expression of LepR (Fig. 4b). Bone marrow-derived macrophages had less expression of CD11b and higher expression of Ly6C compared to the peritoneal macrophages. All cell types, including the DB-1 macrophages had minimal expression of Ly6G, the granulocyte marker, on their surface. DB-1 macrophages displayed MAC-2 in similar amounts as both bone marrow-derived and peritoneal WT and *db/db* macrophages. In contrast, F4/80 was minimally expressed and CD11b was not expressed at all in DB-1 macrophages. Ly6C was expressed by DB-1 macrophages at an intermediate level between that of the bone marrow-derived and peritoneal macrophages (Fig. 4b). These data suggest that the DB-1 macrophages are in the macrophage lineage (Leenen et al. 1990) with high MAC2 expression, lower expression of F4/80 and low levels of Ly6C (Geissmann et al. 2010).

Fig. 4.



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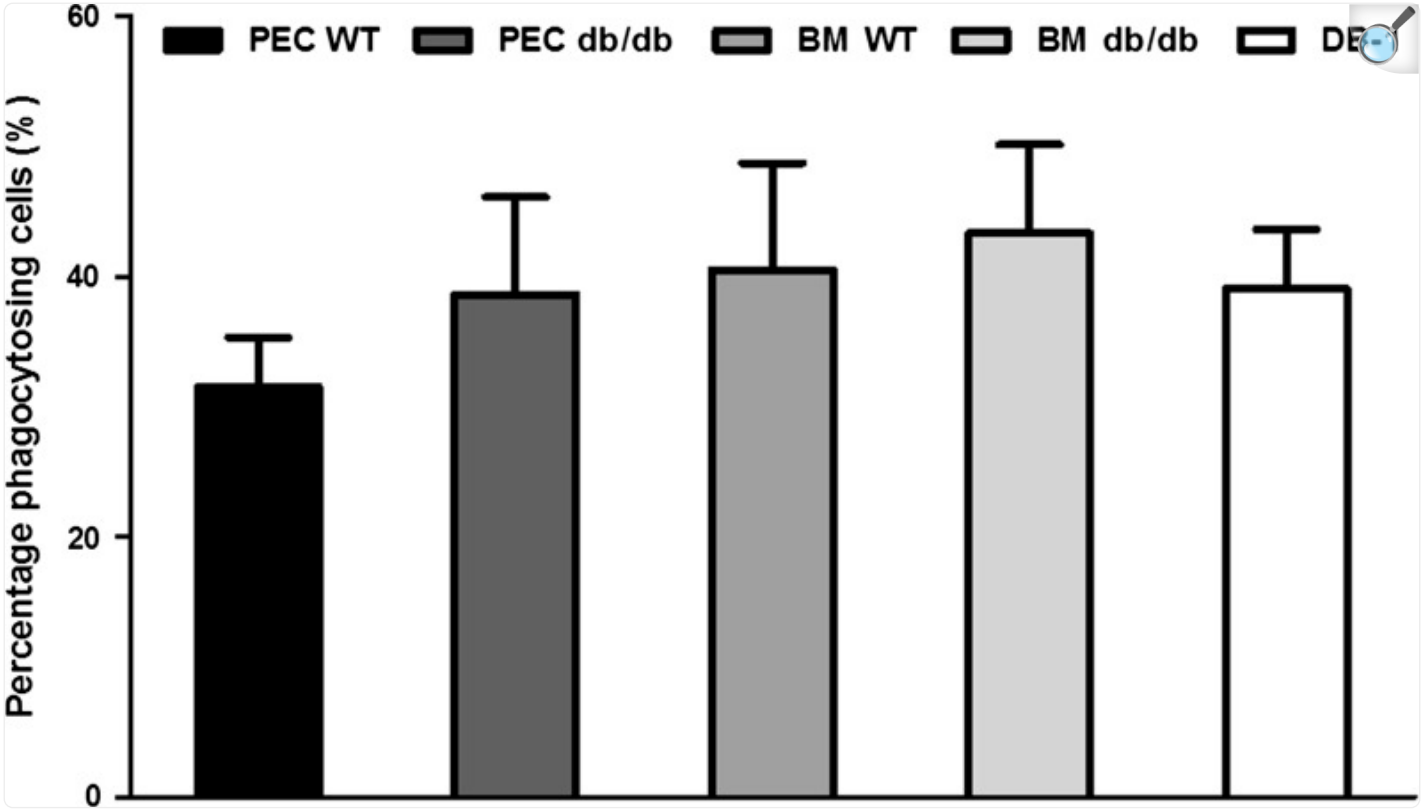
a Flow cytometry analysis of DB-1 cells. Forward-scatter versus Side-scatter (*Column A*) of peritoneal macrophages (*Top two rows*) and DB-1 macrophage cells (*bottom two rows*). Cells were analyzed for CD11b (column B) or F4/80 (column A). Cells were gated (*oval in dot plot* in *column A*) based on forward-scatter and side-scatter. Isotype controls (*rows 1 and 3*, *columns B and C*) were used to set gates to determine the % positive cells. **b** CD11b, MAC-2, F4/80, Ly-6C and Ly6G cell surface expression in peritoneal (PEC WT) macrophages, peritoneal (PEC db/db) macrophages, primary WT bone marrow (BM WT), primary *db/db*

bone marrow (BM db/db) and DB-1 macrophages (DB) using flow cytometry. Data represent means ± SD, from two independent experiments, n = 2–5 replicates per experiment, each n from one or a pool of 2 mice. Significance between different cell types and DB-1 are reported as follows: ***$p < 0.0001$ compared to DB-1, **$p < 0.001$ compared to DB-1, *$p < 0.5$ compare to DB-1. Analysis were performed using one-way ANOVA and Tuckey for post hoc test

Phagocytic activity

To assess whether DB-1 cells display functional macrophage characteristics, we measured the phagocytic activity of DB-1 cells and compared this to that of primary macrophages. We found that all five cell-types—including the DB-1 macrophages—had similar phagocytic indices based on a 1-h phagocytosis assay (Fig. 5, $p > 0.05$).

Fig. 5.



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Phagocytic activity of DB-1, peritoneal (PEC) and bone-marrow (BM) derived WT and *db/db* macrophages after incubation with fluorescent beads. Phagocytic activity assessed via the percentage of phagocytosed beads using flow cytometry. Data represent means ± SD, from one or two independent experiments, n = 3–6 replicates per experiment, each n from one or a pool of 2 mice

DB-1 activation

To test whether DB-1 macrophages respond to a stimulus in a similar fashion as primary macrophages, DB-1 macrophages were stimulated with IFN-γ and transcript levels of *Nos2* were quantified. We found that *Nos2* transcript levels were higher after IFN-γ treatment with levels similar to peritoneal *db/db* macrophages ($p < 0.05$, Table 2). Consistent with transcriptional activation, they also secreted 15, 66 and 131 uM of nitrite in response to 1, 10 and 100 U/ml of IFN-γ, respectively. We also found that DB-1 macrophages secreted 155 ± 48, 385 ± 38 and 538 ± 192 pg/ml of IL-6 in response to LPS, LPS + IFNγ or IFNγ treatments, respectively, compared to low (0 pg/ml) secretion by

unstimulated cells.

Table 2.

Relative transcript level of *Nos2* in DB-1 macrophage cells and peritoneal *db/db* macrophages after stimulation with IFN-γ compared to non-stimulated cells

Cell type	Percent fold up-regulation compared to no treatment group[a]
db/db macrophages	568 (349–1310)[b]
DB-1 macrophages	233 (77–578)[b]

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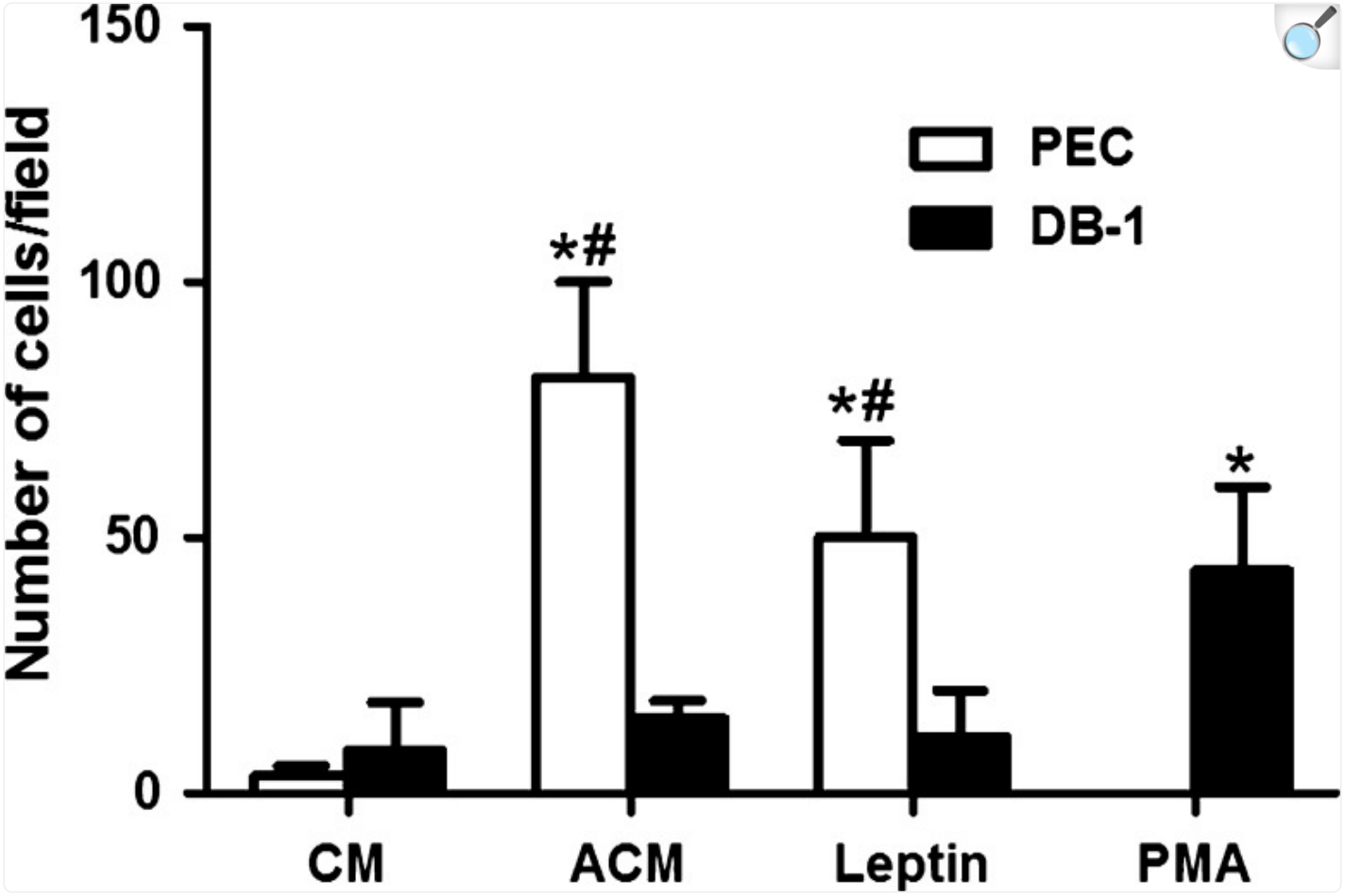
[a]Fold expression was calculated according to Pfaffl (2001) using no treatment values as control group and *ActB* as the reference gene for each macrophage type

[b]Results from 5–8 samples per group, are reported as median (range) expression $p < 0.05$ compared to 100 % expression in control, non-stimulated macrophages

Migration assay

To test whether DB-1 macrophages are responsive to the chemotactic effect of adipocyte conditioned medium or leptin, migration assays were performed using both peritoneal WT and DB-1 macrophages. We found that DB-1 macrophages were activated to migrate when activated with 10 µl of PMA (10 µg/ml; positive control, $p < 0.05$), but that they failed to respond to the chemotactic signals from the ACM as well as the medium supplemented with leptin (Fig. 6). On the other hand, primary WT macrophages had a significant increase in migration when cultured with either the ACM or leptin ($p < 0.01$; comparison to medium alone) with no statistically significant difference between the two media ($p > 0.05$; Fig. 6); suggesting that most of the ACM chemotactic potential is mediated by leptin, and dependent on the presence of a functional LepR.

Fig. 6.



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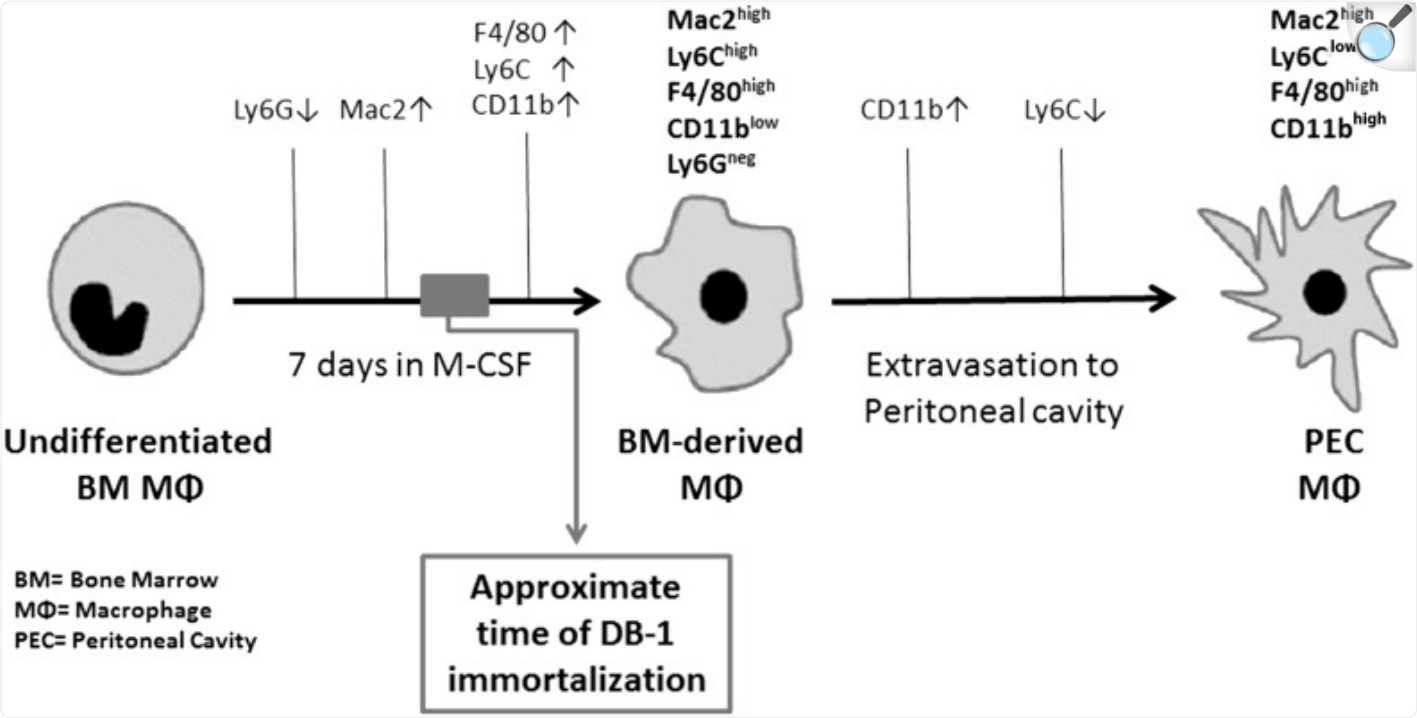
Migration assay of WT peritoneal (PEC) and DB-1 macrophages. In the transwell system the lower chamber was supplemented with control medium (CM), adipocyte conditioned medium (ACM), CM supplemented with 10 ng/ml rm-leptin. To show that DB-1 cells could respond chemotactic stimuli not dependent on the LepR, DB-1 macrophages were stimulated with CM supplemented with 2 μM phorbol 12-myristate 13-acetate (PMA). Values are means ± SD. Data of 1 experiment with n = 3 replicates per treatment group and cell type. *Asterisk* indicates statistically different from same cell type group in control medium (CM) value, $p < 0.05$. [#]Statistically different from DB-1 in the same treatment group, $p < 0.05$

Fig. 7.



Open in a new tab

Placement of DB-1 macrophage cells in the macrophage lineage. The high expression of MAC2 antigen the low expression of F4/80 and Ly6C antigens and the absence of CD11b and Ly6G molecules suggest that DB-1 macrophage cells are early in macrophage differentiation

Discussion

We created a leptin-receptor deficient cell line, DB-1, that has some phenotypic and functional properties characteristic of primary macrophages. It is diploid, has the normal complement of chromosomes, lacks the long isoform of the leptin receptor, and is unresponsive to leptin.

DB-1 cells are in the macrophage lineage based on their selection in macrophage colony stimulating factor over several months' time and because of their phenotypic properties. Indeed, the DB-1 cells have many of the characteristics of ex vivo bone marrow-derived macrophages, thioglycollate-elicited peritoneal WT and primary leptin receptor-deficient macrophages, which the DB-1 cells are hoped to replace in assays. However, their phenotypes are not completely consistent with mouse bone marrow derived macrophages that have been differentiated for 7 days in M-CSF (Leenen et

al. 1994b). Although the DB-1 cells exhibit higher levels of MAC-2 expression and lower levels of Ly6G, indicative of cells in the macrophage lineage (Leenen et al. 1994a), the low expression of F4/80, CD11b and Ly6C would indicate that the cells may be less differentiated. Bone marrow-derived, primary macrophages from *LepR* knock-out mice had a phenotype similar to wild-type bone marrow-derived macrophages and peritoneal macrophages. Therefore, it appears that in response to M-CSF, the bone marrow stem cells began to differentiate into macrophages. MAC2 was the first of these surface molecules to be expressed. Under normal circumstances, the differentiating cells would go on to express F4/80 and Ly6C by 7 days of stimulation with M-CSF (See Fig. 7). DB-1 macrophages don't appear to have reached that stage. Macrophages that extravasate into the peritoneal cavity lose expression of Ly6C and go from low expression of CD11b to high expression of the molecule. DB-1 also do not have that phenotype; suggesting that DB-1 cells are early differentiated macrophages (Chan et al. 1998; Geissmann et al. 2010) (Fig. 7). This hypothesis is supported by the observation that the DB-1 macrophages are responsive to IFN-γ and LPS stimuli; a property of macrophages (MacMicking et al. 1997).

The strong phagocytic activity of the DB-1 cells supports that these cells are in the macrophage lineage as well (Leenen et al. 1986). In our study, the LepR did not appear to contribute to macrophage phagocytosis. The lack of the leptin gene (*Lep*) as well as *Lepr* have been associated with an enhanced susceptibility to infections and a higher mortality rate in rodents following bacterial infection (Ikejima et al. 2005; Mancuso et al. 2002; Ordway et al. 2008; Park et al. 2009). This increased risk of infection has been attributed to a decrease in phagocytic and bactericidal activity of *ob/ob* and *db/db* macrophages in vitro (Loffreda et al. 1998; Mancuso et al. 2002; Park et al. 2009). The difference in phagocytic activity between our DB-1 macrophages and that of *ob/ob* and *db/db* primary macrophages evaluated in other studies might be explained by the fact that we used inert fluorescent beads in our study compared to actual infectious agents (Loffreda et al. 1998; Mancuso et al. 2002; Park et al. 2009). However, we found that DB-1 macrophages's ability to phagocytose fluorescent *Ehrlichia chaffeensis* bacteria was comparable to that of primary macrophages (data not shown). Therefore, the difference between our study and data presented by others may be due to other physiological factors such as a compromised oxidative response (Hsu et al. 2007) in immune cells or inefficient leukotriene production (Mancuso et al. 2002); products that were compromised in primary *db/db* and *ob/ob* macrophages. It is also possible that physiological factors associated with diabetes such as hyperglycemia may have an impact. Park et al. (2009) showed similar infection rates in mice between 8–11 week-old *db/db* mice and WT controls but older (20 weeks and more) *db/db* mice were significantly more susceptible to *S. aureus* infection. The DB-1 cells would not be subject to the physiological regulation of the in vivo macrophages because they are maintained in vitro.

Obesity is associated with an increase in inflammatory macrophage recruitment to the expanding fat mass (Weisberg et al. 2003; Xu et al. 2003). Studies have recognized MCP-1 as the major adipocyte-derived chemokine responsible for this effect (Kanda et al. 2006; Weisberg et al. 2006). Nevertheless, recent studies have questioned this chemokine's role in the enhanced recruitment of macrophages into the adipose tissue of obese mice (Inouye et al. 2007). Leptin is another candidate adipokine/chemokine that might be responsible for this recruitment effect. Indeed, our data show that ACM as well as leptin-supplemented media have the same positive chemotactic effect on WT macrophages. Our DB-1 cell line,

which lacks the functional LepR, failed to respond to both leptin-supplemented as well as adipocyte-conditioned media although our cell line responded to PMA. The differences between the WT macrophages and the DB-1 cells in their responsiveness to leptin can be directly attributed to the absence of LepR on the DB-1 cells which supports the studies of Gruen et al. (2007), who have shown leptin's chemotactic effects at levels as low as 1 pg/ml and illustrates their value in investigating the impact of the LepR in macrophage function. In an era characterized by increasing obesity rates (Ogden et al. 2006), it is of central importance that the immunological consequences of excessive body weight be further elucidated. The newly established DB-1 cell line can serve as an important and reliable tool to study the link between leptin and innate immune function. For example, future studies on the impact of LepR on antigen processing, cell trafficking or the recruitment of other inflammatory cells can be done either in vitro or in vivo. Because the cells are contact inhibited and do not appear to be tumorigenic, we can envision adoptive transfer studies similar to what were done with the C2D macrophage cell line (Ortega et al. 2011; Xie et al. 2010). These cells will be useful to any study investigating the role of the leptin receptor in cell function.

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Footnotes

Tonatiuh Melgarejo and Stephen K. Chapes have contributed equally to this work.

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